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Hemoglobin adducts of aromatic amines: Associations with smoking status and type of tobacco

(molecular epidemiology/protein adducts/aromatic amine carcinogenesis/gas chromatography/mass spectrometry/urinary bladder cancer)

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ABSTRACT **Hemoglobin adducts of 15 aromatic amines were determined in nonsmokers and smokers of blond- or black-tobacco cigarettes living in Turin, Italy. The subjects were all males age 55 or less and were representative of the population previously examined in a case/control study of bladder cancer. 4-Aminobiphenyl adduct levels were found to be significantly different in the three groups, and the differences were approximately proportional to the relative risk of each group. Adjustment for age and cigarette consumption did not materially influence the differences. A significant correlation of adduct levels with cigarette consumption was also observed for all smokers as well as for smokers of blond tobacco. Other amines for which significant differences between smokers and nonsmokers were observed were 3-aminobiphenyl, 2-naphthylamine, *o*- and *p*-toluidine, 2,4-dimethylaniline, and 2-ethylaniline. Some of these amines are human bladder carcinogens, and their occurrence in blood as hemoglobin adducts is evidence for their metabolic activation. Thus, by a combination of traditional epidemiological methods and modern chemical analyses, we have provided evidence for a biochemical basis for the often observed association between cigarette smoking and bladder cancer.**

Cigarette smoking is a major cause of cancer of the urinary bladder. In countries where a long history of cigarette consumption exists, the proportion of bladder cancers attributed to this risk factor is estimated to be about 50% for men and 30% for women (1–3). The relative risk for bladder cancer among cigarette smokers ranges in most reports from 1.5- to 3.0-fold higher than among nonsmokers, with higher values reported in certain populations (4). It has been hypothesized that the carcinogenicity of tobacco smoke for the bladder may be due to the presence of various aromatic amines, including the known human bladder carcinogens 4-aminobiphenyl and 2-naphthylamine (5).

Current evidence indicates that aromatic amines exert their carcinogenic effects on the bladder only after a number of metabolic and distribution steps. The critical activation to an electrophilic intermediate is effected in the liver through N-hydroxylation of the parent amine to the hydroxylamine. This is followed by transport to the bladder, where reaction with DNA is thought to initiate tumorigenesis (6–8). We have developed and reported (9–11) a method to quantify the time-weighted average hydroxylamine production over a period of several months from exposure to environmental aromatic amines. The technique depends upon measurement of the covalent sulfinamide adduct of the β-chain cysteine-93 residue formed by interaction of aromatic hydroxylamines with hemoglobin. Although stable *in vivo*, this adduct can be broken down *in vitro* to regenerate the parent amine and a hemoglobin molecule containing cysteine sulfinic acid. Accurate and sensitive quantification of the adduct is accomplished by mass spectrometric analysis of the regenerated amines after separation by gas chromatography. Previous studies established that the hemoglobin adducts of 4-aminobiphenyl were higher in a group of cigarette smokers than in nonsmoking subjects (10). The present work describes results of a study conducted on residents in the city of Turin, Italy, in which hemoglobin adducts of 4-aminobiphenyl and other aromatic amines were measured in a population previously studied with regard to bladder cancer risk (4). A putative etiologic role for this class of compounds in the bladder carcinogenicity of tobacco smoke could thus be examined.

METHODS

Subjects. Male volunteers were recruited at a blood donor center in the city of Turin, Italy, to donate an additional 10-ml blood sample and fill out a questionnaire on their smoking history and other parameters such as occupation. During two collection periods, 87 samples were collected from 25 nonsmokers, 40 blond-tobacco smokers, 18 black-tobacco smokers, and 3 mixed blond/black-tobacco smokers. Classification of tobacco type was done as described (4) based on whether the particular brand of cigarette is composed of air-cured tobacco (black) or flue-cured tobacco (blond). The mixed blond/black-tobacco smokers were grouped with the blond-tobacco users. The subjects were age 55 or less and are representative of the resource population previously used in a case/control study for bladder cancer (4).

Measurement of aromatic amine–hemoglobin adducts. Erythrocytes were washed with saline, frozen, and then coded for later analysis. All analyses were performed without prior knowledge of sample identity.

The detailed procedure for quantification of 4-aminobiphenyl-hemoglobin adducts has been described (10, 11) but is discussed briefly here. Isolated hemoglobin is purified by dialysis and then hydrolyzed with base to release the parent amine. After extraction into hexane, the amine is derivatized to form the pentafluoropropionamide. Quantification is accomplished by comparison of peak areas produced by the analyte and an internal standard (4'-fluoro-4-aminobiphenyl) upon capillary gas chromatography using negative-ion chemical-ionization mass spectrometry for detection. Detection of other aromatic amines was achieved by monitoring ions corresponding to the respective pentafluoropropionamides. Two additional internal standards, [*ring*-$^2H_5$]aniline (d_5-aniline) and 5-fluoro-2-naphthylamine, were used for quantification. Precision of the method is somewhat less (±20%) for the analysis of the substituted anilines than was seen with the analysis of 4-ABP (±5–10%). This is most likely caused

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by the use of [*ring*-$^2H_5$] aniline as the internal standard for the substituted anilines, with resultant differences in volatility of the derivatives and in recovery of the amines. This problem would be alleviated through the use of more internal standards that are more closely analogous to the various substituted anilines (toluidines, ethylanilines, dimethylanilines). Nonetheless, [*ring*-$^2H_5$]aniline is considered to be an acceptable internal standard for the limited application being considered in this report—that of whether the various substituted anilines are correlated with smoking status or tobacco type.

Statistical Analysis. Adjustments of adduct levels by age of the subjects or by amount smoked as continuous variables were done with the least-squares method using a linear model. Correlations with smoking status (see Table 3) were determined by comparing the 25 nonsmokers with the 61 smokers. Statistical significance was determined with a Wilcoxon two-sample test (normal approximation with a continuity correction of 0.5). Correlations with tobacco type were determined by comparing the 43 smokers of blond tobacco with the 18 smokers of black tobacco. Description of the specific methods used are given in ref. 12.

RESULTS AND DISCUSSION

Hemoglobin Adducts of 4-Aminobiphenyl and 3-Aminobiphenyl. Histograms of the 4-aminobiphenyl-hemoglobin adduct levels in the three groups (nonsmokers and smokers of blond and black tobacco) are presented in Fig. 1. Distribution of adduct levels in the three groups clearly shows that both smoking groups have increased adduct levels compared to nonsmokers, and that smokers of black tobacco have higher adduct levels than those of smokers of blond tobacco. Differences among these three groups were found to be statistically significant ($P = 0.0001$) by analysis of variance. The unadjusted means and standard errors of the 4-aminobiphenyl-hemoglobin adduct levels in the three groups are listed in Table 1. The highest levels of the adduct (>600 pg/g of hemoglobin) were found in two smokers of filtered black tobacco cigarettes. The mean value in blond-tobacco smokers (176 pg/g) is similar to that found in previous studies in the United States (154 pg/g; ref. 10), where blond tobacco is smoked primarily.

The age distribution of subjects by type of tobacco showed that a higher proportion of older subjects smoked black tobacco. It was also observed that the black tobacco smokers consumed more cigarettes per day than the smokers of blond tobacco. Adjusted for smoking, mean adduct levels in all smokers were 135 (SEM = 19) for smokers of ages less than 35, 167 (SEM = 18) for smokers of ages 35–44, and 188 (SEM = 13) for smokers of ages more than 45. Thus, a trend to higher adduct levels with increasing age was evident. It was not statistically significant ($P = 0.099$), and adjustment by age had no material effect on the estimates (see Table 1).

The amount smoked per day was highly correlated ($P = 0.0015$) with adduct level for all smokers by analysis of variance using a linear model. This dose–response relation-

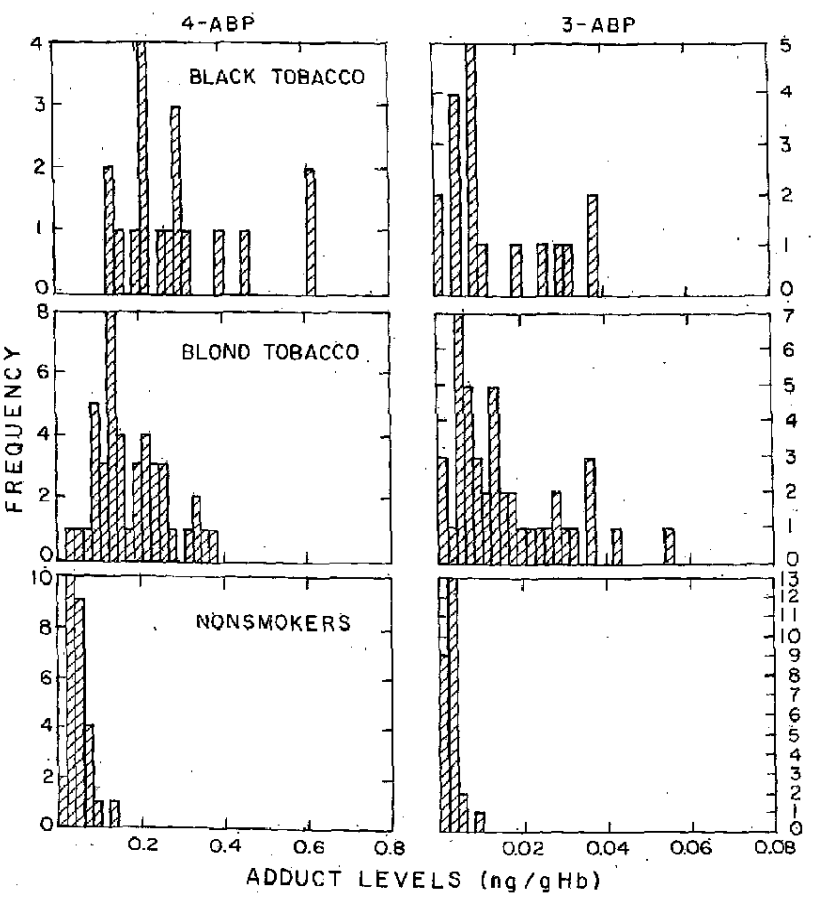

FIG. 1. Hemoglobin adduct levels of isomeric aminobiphenyls in smokers of black tobacco, smokers of blond tobacco, and nonsmokers. Average daily cigarette consumption was 21.2 in the first group and 16.8 in the second. The difference in consumption between the two groups is not statistically significant. 4- and 3-ABP, 4- and 3- aminobiphenyl.

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Table 1. Influence of age and cigarette consumption on 4-aminobiphenyl (4-ABP)-hemoglobin adduct levels

Group (*n*)	Mean adduct level, pg of 4-ABP per g of Hb (SEM)			
	Unadjusted	Adjusted*		
		Age	Amount smoked	Simultaneous
Nonsmokers (25)	51 (4)	—	—	—
Smokers				
Blond tobacco (43)	176 (12)	186 (17)	165 (16)	165 (14)
Black tobacco (18)	288 (34)	277 (25)	256 (26)	248 (23)

Subjects were grouped according to reported smoking status and the type of tobacco smoked. The three mixed black/blond-tobacco smokers were included in the blond-tobacco category. The number of subjects is in parentheses.
*Mean adduct levels were adjusted by the least-squares mean method.

ship was examined within each tobacco type and found to be statistically significant for blond tobacco ($P = 0.0074$). A trend within the black-tobacco smokers was evident but was not statistically significant ($P = 0.183$). Within each consumption category, the black-tobacco smokers showed adduct levels that were 40–50% higher than levels of blond-tobacco smokers (see Table 2). This result is in agreement with a report that black-tobacco cigarettes produce more 4-aminobiphenyl in the mainstream smoke than do blond-tobacco cigarettes (13).

None of the subjects was involved in occupations known to be associated with aromatic amine exposure. Furthermore, no occupation was associated with any of the three comparison groups (nonsmokers and blond- or black-tobacco smokers). Thus, occupation is unlikely to be a confounding factor.

The amount-adjusted mean adduct levels listed in Table 1 indicate that 4-aminobiphenyl-hemoglobin adducts were 3.2 times higher in blond-tobacco smokers as a group compared to nonsmokers. Similarly, in black-tobacco smokers the mean adduct levels were 5 times higher. The relative risks for bladder cancer historically seen in this population (4) follow a similar pattern: the risk is higher for smokers of either tobacco type compared to nonsmokers and is highest in smokers of black tobacco. Thus, there is an association between adduct levels of 4-aminobiphenyl and relative risk for bladder cancer,[¶] which is consistent with the hypothesis that there is an etiologic role for 4-aminobiphenyl in this disease. The predictive value of adduct levels in an individual is as yet undetermined, since many additional factors may influence tumorigenesis. These could include exposure to other carcinogens as well as to promoting agents, physiological variables that affect DNA adduct formation such as urine pH and frequency of micturition, effectiveness of DNA repair mechanisms, and immunological status.

The adduct levels for 3-aminobiphenyl were much higher in the blood of smokers than nonsmokers, as illustrated in Fig. 1. The mean adduct levels for the blond-tobacco smokers (14 pg/g of hemoglobin, SEM = 2) and black-tobacco smokers (13 pg/g, SEM = 3) are 12 and 11 times greater, respectively, than the mean adduct level for the nonsmokers (1.2 pg/g, SEM = 0.4). Although the difference between smokers and nonsmokers is statistically significant ($P < 0.0001$), that between blond- and black-tobacco smokers is not. A statistically significant ($P = 0.02$) relationship between adduct level and the number of cigarettes consumed was observed among blond-tobacco smokers but not among black-tobacco smokers. Thus, with both tobacco types combined, there was a dose–response relationship of borderline significance ($P = 0.06$). The difference between smokers and nonsmokers was much greater for 3-aminobiphenyl than it was for 4-aminobiphenyl. In contrast, the ratios of the two amines in sidestream to mainstream smoke are virtually identical (13). Thus, exposure to 3-aminobiphenyl appears to be highly tobacco specific. This is less so for 4-aminobiphenyl, which suggests that there are other environmental sources for the adducts of this isomer found in nonsmokers.

Substituted Anilines and 2-Naphthylamine. The unadjusted mean adduct levels of the other arylamines observed in the three groups are listed in Table 3, along with the standard errors. Also listed in Table 3 are the P values for the association of adduct levels with either smoking status (smoker versus nonsmoker) or tobacco type (black tobacco versus blond tobacco). Five amines in addition to 4- and 3-aminobiphenyl appear to be associated with smoking status: 2-naphthylamine, *o*-toluidine, *p*-toluidine, 2-ethylaniline and 2,4-dimethylaniline. Three of these amines also show an association with tobacco type: *o*-toluidine, *p*-toluidine, and 2,4-dimethylaniline. Adduct levels of most of the substituted anilines did not appear to exhibit significant differences between smokers of either tobacco type and nonsmokers. Thus, the chief exposure to these amines may be unrelated to smoking. Similar results were observed in a recent study (14) in which the levels of aniline and the three toluidines measured in the urine of smokers were not significantly different from those of nonsmokers.

CONCLUSION

The significance of epidemiological investigations is enhanced by accurate estimates of exposure to the putative carcinogenic agents. The field of molecular epidemiology is emerging by combining new biochemical indicators of exposure with traditional epidemiological methods (15–19). Various chemical markers of human exposure to carcinogens have been measured in recent years (14, 20–26). The results of this study indicate that measurement of the hemoglobin adducts of 4-aminobiphenyl and other aromatic amines may be useful in examining the biochemical basis for the increased risk for bladder cancer in smokers.

Table 2. Dose–response relationship for age-adjusted 4-aminobiphenyl (4-ABP)-hemoglobin adduct levels

Cigarettes smoked per day	Blond tobacco	Black tobacco
<10	124 (9)	— (0)
10–19	155 (15)	223 (6)
20+	216 (19)	321 (12)

The fitted line for all data was the 4-ABP-Hb adduct level (ng of 4-ABP per g of Hb) = 0.12 + 0.005 × cigarettes per day (CPD) for all smokers. For blond- and black-tobacco smokers, the lines were 0.112 + 0.0039 × CPD and 0.218 + 0.003 × CPD, respectively. Values in parentheses are the number of subjects in each group (*n*).

[¶]A similar association between urinary excretion of mutagens and relative risk also has been observed in this population (C. Malaveille, P.V., J. Esteve, H. Ohshima, G. Brun, A. Hautefeuille, P. Gallet, G. Ronco, B. Terracini, and H. Bartsch, unpublished data).

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Table 3. Hemoglobin adduct levels of 2-naphthylamine and alkyl anilines: Associations with smoking status and tobacco type

Aromatic amine	Mean adduct level, pg of amine per g of Hb (SEM)			*P* for association	
	Nonsmokers	Smokers			
		Blond tobacco	Black tobacco	Smoking status	Tobacco type
2-Naphthylamine	11.6 (1.1)	17.2 (1.8)	21.3 (4.9)	0.002	0.90
o-Toluidine	188 (19)	290 (19)	329 (22)	<0.0001	0.039
m-Toluidine	1141 (138)	1097 (108)	1140l (138)	0.98	0.56
p-Toluidine	209 (24)	306 (35)	415 (73)	0.004	0.03
2,5-Dimethylaniline	50 (6)	67 (10)	70 (14)	0.45	0.63
2,4-Dimethylaniline	40 (5)	73 (10)	114 (17)	0.0005	0.008
2,6-Dimethylaniline	264 (90)	86 (15)	98 (30)	0.14	0.82
2,3-Dimethylaniline	52 (8)	56 (11)	64 (19)	0.55	0.73
3,5-Dimethylaniline	931 (12)	112 (31)	135 (31)	0.70	0.09
3,4-Dimethylaniline	47 (11)	46 (11)	65 (19)	0.71	0.045
2-Ethylaniline	38 (3)	70 (10)	80 (12)	0.0015	0.16
3-Ethylaniline	102 (16)	115 (22)	129 (34)	0.89	0.63
4-Ethylaniline	92 (17)	77 (13)	111 (41)	0.25	0.50

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